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Heavy metal toxicity to bacteria – Are the existing growth media accurate enough to determine heavy metal toxicity?

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HIGHLIGHTS

- A new bacterial growth medium containing low levels of metal-chelates was formulated.
- This medium has high free metal ion activity and more suitable to determine metal toxicity.
- This medium provides a viable option for the study of metals–bacteria interactions.

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ABSTRACT

A new minimal medium was formulated considering the limitations of the existing media for testing heavy metal sensitivity to bacteria. Toxicity of cadmium and copper to three bacteria was investigated in the new medium and compared with three other media commonly used to study the effect of the toxic metals. Based on speciation data arrived at using ion-selective electrodes, the available free-metal concentration in solution was highest in the MES-buffered medium. This finding was strongly supported by the estimated EC_{50} values for the metals tested based on the toxicity bioassays. The free-ionic cadmium and copper concentrations in the medium provide more accurate determination of metal concentrations that affects the bacteria, than with most of other existing media. This will avoid doubts on other media and misleading conclusions relevant to the toxicity of heavy metals to bacteria and provides a better option for the study of metal–bacteria interactions.

1. Introduction

Toxicity of various heavy metals to bacteria isolated from numerous habitats in the terrestrial environment has been extensively studied for many years. Many different approaches have been used to study the metal toxicity to bacteria either as single isolates (Babich and Stotzky, 1977; Keeling and Cater, 1998; Tangaromsuk et al., 2002; Madhaiyan et al., 2007), or mixed cultures (Saeki et al., 2002; Zhang et al., 2003) in solution, or in communities in soil, water, etc. (Diaz-Raviña and Bååth, 1996; Rajapaksha et al., 2004; Oliveira and Pampulha, 2006), to mention a few.

Using widely different bacteriological culture media, a broad range of metal concentrations have been reported on the tolerance of heavy metals by bacteria from various environments (Duxbury and Bicknell, 1983; El-Aziz et al., 1991; Martensson and Torstensson, 1996; Malik and Jaiswal, 2000; Roane and Pepper, 2000; Amoroso et al., 2002; Renella et al., 2003; Yilmaz, 2003; Piotrowska-Seget et al., 2005; Chen et al., 2006; Sprocati et al., 2006; Abou-Shanab et al., 2007; Kim et al., 2007). The main basis for the choice of a specific medium was its ability to encourage growth of the specified bacteria or group of bacteria in that medium (Angle and Chaney, 1989; Richards et al., 2002; Chen et al., 2006; Miranda and Rojas, 2006; Congeevaram et al., 2007). The media were supplemented with different concentrations of nutrient metal salts and were inoculated with relevant bacterial isolate or group of isolates. The growth was then measured by various parameters such as, turbidity, biomass, and enzyme activities to determine the sensitivity by different criteria, viz., EC_{50}, MIC (minimum inhibitory concentration), LD_{50}, etc., after specified time of incubation at a specified temperature either in solid (Hassen et al., 1998; Saeki et al., 2002) or liquid media (Hassen et al., 1998; Clausen, 2000).

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In most studies, bacteriological culture media that were used contained undefined organic components such as nutrient broth (Hassen et al., 1998), peptone, tryptone, and yeast extract (Babich and Stotzky, 1977; Konopka et al., 1999; Chen et al., 2006; Kim et al., 2007), and beef extract (Malik and Jaiswal, 2000). Media with defined chemical composition (Martensson and Torstensson, 1996; Amoroso et al., 2002; Legatzki et al., 2003) but with very high amount (2–10 g L^{-1}) of carbon source, e.g. glucose, gluconate, mannitol, etc. have also been used. Use of the above mentioned media resulted in very high levels of metal tolerance by the bacteria grown in those media (Megharaj et al., 2003). The complexation or chelation of metals to the unspecified organic constituents in the medium and the lack of stability constants of metal–organic complexes might have resulted in an overestimate of the metal tolerance levels of bacteria under investigation.

Another important component of a bacteriological culture medium is the pH buffer. In culture media pH buffers are present at higher concentrations than other medium components. Phosphate is the most common buffer used in microbiological media (Brynhildsen et al., 1988; Farrell et al., 1993; Martensson and Torstensson, 1996). Addition of phosphate to the media can cause precipitation of heavy metal ions as insoluble phosphates reducing their bioavailability and toxicity. The phosphate buffers were also substituted with Zwitterionic biological buffers, so called 'Good's buffers' (Good and Izawa, 1972; Good et al., 1996), such as MES [2-(n-morpholino) ethane sulfonic acid, p*K*a = 6.15, Angle and Chaney, 1989], PIPES [1,4-piperazinediethanesulfonic acid, p*K*a = 6.8, Hoffman et al., 2005], HEPES [4-2-hydroxyethyl-l-piperazine-ethanesulfonic acid, p*K*a = 7.55, Knotek-Smith et al., 2003; Teitzel and Parsek, 2003), MOPS [3-N-morpholino-propansulfonic acid, p*K*a = 7.2, Riether et al., 2001] and TRIS [trishydroxy methyl amino methane, p*K*a = 8.06, Mergeay et al., 1985; Rubikas et al., 1997; Legatzki et al., 2003; Abou-Shanab et al., 2007; Madhaiyan et al., 2007] to optimize the metal bioavailability in the culture media (Roane and Pepper, 1999).

Besides the presence of phosphate as buffer and undefined organic compounds, the main drawback in the use of most of these media is the small amount of the free metal ion activity in the medium. The free metal ion activity determines the microbial response to the metal toxicity and the free ion activity model (FIAM) is utilized uniformly in estimating metal uptake and, thus, metal toxicity (Campbell, 1995). Metal in aqueous phase can be present in the medium in many different forms (Hughes and Poole, 1991; Traina and Laperche, 1999), depending on chemical composition and the pH of the medium. However, limited attention has been paid to the nature of metal species present in the medium in understanding metal uptake and toxicity (Hughes and Poole, 1991). Thus, it is essential to determine the free metal ion activity, which is the most critical factor that determines the metal toxicity to bacteria, before making the choice of a bacteriological medium (Mergeay, 1995).

Computer speciation modeling of the bioassay media conducted in parallel with the toxicity experiments might be useful in interpreting the biological effects of the heavy metals (Farrell et al., 1993). The geochemical modeling software like MINTEQL, GEOCHEM-PC (Hughes and Poole, 1991; Farrell et al., 1993; Twiss et al., 2001; Teitzel and Parsek, 2003; Hoffman et al., 2005) is a very powerful tool in predicting metal speciation which can be related to bioavailability at equilibrium; however, the computer simulation calculations apply only to systems in thermodynamic equilibrium and do not reflect the slow reactions that take place in the medium or microbial transformations of metallic species during the microbial growth (Hughes and Poole, 1991). Indeed, many of the microbiological media are rarely at equilibrium (Hoffman et al., 2005). The ion selective electrodes (ISE) offer an excellent alternative for estimating the free metal ion concentration (>10^{-7} M).

The main objectives of this study were (1) to formulate a new minimal medium which provides optimum free heavy metal ion concentration in solution resulting in maximum toxicity to bacteria and (2) to compare the suitability of existing media to determine heavy metal toxicity to bacteria isolated from 3 different pristine soils which have cadmium and copper levels below the Australian National Environment Protection (Assessment of site contamination) Measure (NEPM) ecological investigation levels (EILs) for the metals in soil (NEPM, 1999).

2. Materials and methods

2.1. Comparison of existing bacteriological culture media

Three bacteriological media, which are commonly in use in the toxicological studies, were used in this study to compare the effect of the media components on the availability of the heavy metals added to the media. All the media were prepared using MilliQ water.

TRIS minimal medium (pH 7.0) (Mergeay et al., 1985) consists of the following components (g L^{-1}): TRIS HCl, 6.06; NaCl, 4.68; KCl, 1.49; NH_4Cl, 1.07; Na_2SO_4, 0.43; $MgCl_2{\cdot}6H_2O$, 0.20; $CaCl_2{\cdot}2H_2O$, 0.03; Na_2HPO_4, 0.04, Fe(III)NH_4 citrate, 0.005, glucose, 2.00; and 1 mL of the SL7 oligoelement solution (Biebl and Pfenning, 1981). PTY80 broth (pH 6.5) (Konopka et al., 1999) consists of the following components (g L^{-1}): peptone, 0.08; tryptone, 0.08; yeast extract, 0.08; and 2-(N-morpholino) ethanesulfonic acid (MES), 1.95. Trypticase soy broth (pH 7.3) consists of the following components (g L^{-1}): Trypticase peptone 17; peptone 3; NaCl 5; K_2HPO_4 2.5; and glucose 2.5. The media were prepared according to the manufacturer's instructions (Oxoid). The pH of the media was adjusted and the media were sterilized by autoclaving.

2.2. Formulation of a new minimal bacteriological medium

The MES buffered minimal medium (MBMM) was formulated by considering the nutritional requirements of bacteria; the nutrients were added in minimum concentrations to obtain good inoculum and to result in fair amounts of free metal ion concentration. The constituents (g L^{-1}) of the medium (pH 6.4) are as follows: 2-(N-morpholino) ethanesulfonic acid (MES), 1.95; Na_2HPO_4, 0.01; NH_4Cl, 0.05; $CaSO_4$, 0.14; $MgSO_4{\cdot}7H_2O$, 0.24; KCl, 0.02; $FeSO_4{\cdot}7H_2O$; 0.004, and 1 mL of SL7 trace element solution (Biebl and Pfenning, 1981) consisting of the following constituents (mg L^{-1}): $ZnCl_2$, 70; $MnCl_2{\cdot}4H_2O$, 100; H_3BO_3, 60; $CoCl_2{\cdot}6H_2O$ 200; $CuCl_2{\cdot}2H_2O$, 20; $NiCl_2{\cdot}6H_2O$, 20; $NaMoO_4{\cdot}2H_2O$, 40; and 1 mL of HCl (25%). Glucose (0.2%) as the carbon source was also added to the medium.

2.3. Speciation of Cd and Cu

The speciation of Cd and Cu in the media used was arrived at using a software geochemical equilibrium speciation model (Visual MINTEQ, Gustafsson, 2005) and with Ion Selective Electrodes (ISEs) (Phoenix Electrode Co.). The Cd^{2+} and Cu^{2+} standards were prepared in MilliQ water using the nitrate salts (Sigma–Aldrich) to obtain concentrations between 0.05 and 10 mg L^{-1} and filtered with 0.45 μm Millipore sterilized filters. 2 mL of 5 M $NaNO_3$ was added to each standard to keep the ionic strength constant.

2.4. Dose response experiments using bacterial isolates

Three bacteria belong to the genus *Bacillus* (*Bacillus megaterium*, *Bacillus thuringiensis*, and *Bacillus simplex*) were selected based on the identification of the bacteria in prestine soils. Inocula were